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Hematologic effects of Escherichia coli O157:H7 and Shiga-like toxin 1

Denise Stephanie Wunn
Iowa State University

Hematologic effects of *Escherichia coli* O157:H7 and Shiga-like toxin 1

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Denise Stephanie Wunn

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DEDICATION

This thesis is dedicated to my family, in recognition of the support (financial and spiritual) that has seen me through the most difficult times in my life. Thanks to my parents Duane and Dolores, my sisters Dianne Cancian and Deb Bode and their families. Without you, none of this would have been accomplished.

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LIST OF SYMBOLS AND NOMENCLATURE

2D ELP Two-dimensional electrophoresis

BSA Bovine serum albumin

EC Endothelial cell

Ehx Enterohemolysin

ER Endoplasmic reticulum

EspP Extracellular serine protease, plasmid encoded

FITC Fluorescein isothiocyanate

G_1/S Period between 1st growth phase and DNA synthesis phase of cell cycle

Gb_3 Globotriaosyl ceramide

Gb_4 Globotetraosyl ceramide

HBSS Hank's balanced salt solution

HlyA α-Hemolysin

HUS Hemolytic Uremic Syndrome

HUVEC Human umbilical vein endothelial cells

IL-1 Interleukin-1

KDEL Amino acid sequence lysine, aspartic acid, glutamic acid, leucine

LPS Lipopolysaccharide

PAI-1 Plasminogen activator inhibitor-1

PBS Phosphate buffered saline

RBC Red blood cell

RTX Repeats in toxin

SDS-PAGE Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

STEC Shiga toxin producing *E. coli*

STX Shiga-like Toxin

TNF_α Tumor necrosis factor α

TTP Thrombotic thrombocytopenic purpura

uPA Urinary plasminogen activator

vWF von Willebrand factor

ABSTRACT

The hemolytic uremic syndrome (HUS) of children was originally described as a triad of acute renal failure, fragmented red blood cells (RBCs) and thrombocytopenia. This syndrome is most often associated with infection with *Escherichia coli* O157:H7 and the shiga-like toxin (STX) it produces. One of the proposed pathogenic mechanisms of RBC fragmentation was damage to RBCs as they were forced through fibrin thrombi that occur in the renal microvasculature. However, direct damage to RBC by *E. coli* toxins may also be involved. In order to better understand the mechanistic basis of RBC fragmentation during HUS, two studies were carried out. The first examined the utility of computerized image analysis and a single shape factor equation ({Crofton perimeter^2}÷{4*π*Area}) to quantify the degree of poikilocytosis in calves infected with *E. coli* O157:H7. The percentage of cells with different shape factor scores were compared in calves prior to and after infection with *E. coli* O157:H7. Cell shape was also manually scored by two investigators. No significant increases in poikilocytes were detected using either image analysis or manual scoring in calves after infection with *E. coli*. Crenation of calf RBCs caused the greatest discordance between image analysis and manual scores. The tendency of normal calves to have crenated RBCs impaired the usefulness of the image analysis procedure and a single shape factor equation. The second study examined the direct effects of STX on human RBCs *in vitro* and found no significant differences in osmotic fragility or RBC membrane proteins as evaluated with polyacrylamide gel electrophoresis. The effects of STX on RBC adhesion to confluent layers of cultured endothelial cells was studied using a gravity adherence assay. STX was incubated with human microvascular endothelial cells and uptake of the toxin by the

endothelial cells was confirmed by immunofluorescence. RBCs were added to the chamber and incubated at 37°C for 30 minutes. No increase in RBC adherence to endothelial cells was observed after incubation with STX. The mechanism of human RBC fragmentation is likely an indirect effect of STX. Combinations of *E. coli* toxins, including lipopolysaccharide, may also be involved in the pathogenesis of RBC fragmentation.

GENERAL INTRODUCTION

Literature Review

In 1955, Swiss hematologist Conrad von Gasser published the first report of five children dying of what was described as a hemolytic uremic syndrome (HUS). The cases were identified by the now classic triad of thrombocytopenia, renal failure and fragmented red blood cells (RBCs).[1] For several years after the initial report, there were attempts to understand the cause of HUS. In 1983, Karmali made important observations by recovering shiga-like toxin (STX) and/or shiga-like toxin producing *Escherichia coli* (STEC) from the feces of children with HUS.[2, 3] It is now widely believed that the effect of STX on the endothelial cell, particularly in the renal glomeruli, is the principal lesion that results in HUS.[4]

HUS is a leading cause of acute renal failure in American children. Approximately 73,000 cases are reported each year with 65 fatalities.[5] Since the requirement for disease notification has begun, there has been a steadily increasing trend of cases of HUS in the United States.[6] It is estimated that HUS causes the death of approximately 250 children each year with permanent renal dysfunction a problem for nearly one-third of the survivors.[7] While most cases of HUS occur in children under five years of age, elderly adults are also identified as having increased susceptibility to the disease with several outbreaks being reported in nursing homes.[8] Many cases of HUS have been associated with consumption of contaminated ground beef that was improperly prepared,[7,9] although other methods of transmission have been reported.

After exposure to *E. coli* O157:H7, there is a latent period of 1 to 9 days, followed by severe abdominal cramps as the usual initial complaint.[8] Watery diarrhea rapidly follows and

may progress to bloody diarrhea in 35 to 90 percent of the cases.[7] Laboratory evaluations usually reveal leukocytosis with a left shift. Histologically, the colon lesions are often characterized by submucosal edema, hemorrhage and deposition of fibrin, with less frequent capillary thrombosis, ulceration or suppurative inflammation.[8] Renal histopathology often reveals glomerular thrombosis, necrosis or, in adults, intimal proliferation. Severe cases may undergo complete renal cortical necrosis.[10] Resolution of the diarrhea often occurs within one week during uncomplicated infections, however, approximately 6% of the patients develop HUS 2 to 14 days after the onset of diarrhea. Factors that tend to be indicative of progression to HUS include high fever, age and leukocytosis with a left shift at the time of presentation.[7]

Shiga-toxins produced from *E. coli* belongs to a family of toxins, the prototype of which was named for the bacterium *Shigella dysenteriae*. Because this family of toxins was originally studied on Vero cells (kidney cells from the African green monkey), the synonym verotoxin is common throughout the historical literature and remains in use today.[11] Human infections with STEC are usually associated with STX 1 or STX 2, with STX 2 most often associated with cases of HUS.[12] STX 1 is nearly identical to the toxin produced by *Shigella*; STX 2 is approximately 60% identical with STX 1. STX 2e is the cause of pig edema disease and has been found to have approximately 90% sequence identity with STX 2.[13] STX 1 and 2 are encoded on a bacteriophage.[14] Members of the STX family exhibit similar protein structure, composed of a catalytically active A subunit and a receptor binding B subunit. The A subunit is approximately 33 kDa; the B subunit forms a pentamer from interlocking monomers of 7.5 kDa.[15] The central core of the B subunit pentamer interacts with the alpha-helical carboxy terminus of the A subunit and maintains the stability of the

holotoxin.[16] Assembled toxin is not actively secreted from the bacterium; lysis of the cell must occur before toxin is released.[14]

The Shiga family of toxins bind neutral glycolipids on the surface of host cells. The specific receptor for both STX 1 and 2 is globotriaosyl ceramide (Gb_3, Galactose $\alpha[1\rightarrow4]$-Galactose $\beta[1\rightarrow4]$ glucosyl ceramide).[13] In addition to the name Gb_3, this glycolipid has been called the Burkitt's lymphoma antigen, CD77 and human blood type P^k.[17] In contrast, STX 2e binds Gb_3, but its preferred receptor is globotetraosyl ceramide (Gb_4, N-acetylgalactosamine $\beta[1\rightarrow3]$ Galactose $\alpha[1\rightarrow4]$-Galactose $\beta[1\rightarrow4]$ glucosyl ceramide).[13] The Phe30 site of the B subunit of STX 1 has been shown to be an important site for interaction between the toxin and its receptor. Other sites, such as the Trp34 site, have been studied and may yet prove important for toxin interaction with its receptor.[16] In addition to presentation of the Gb_3 glycolipid, the length and degree of saturation of the remainder of the lipid alters the ability of the toxin to bind to the receptor. Chain lengths between 14 and 22 carbons and higher degrees of chain unsaturation have been show to maximize STX 1 binding. It is proposed that the chain length of the lipid influences the three-dimensional structure of the receptor and the bound toxin and ultimately affects the stability of the receptor-toxin complex.[13]

The shiga toxin family has a unique, and still not fully understood means of intracellular transport and activation. Once the toxin is bound to Gb_3, it is internalized via clathrin dependent mechanisms. This is in contrast to cholera and tetanus toxins that also bind lipid receptors but are internalized without utilizing clathrin.[18] Once internalized, the majority of the toxin is transported to lysosomes and undergoes degradation; approximately 10% of the toxin is transported to the Golgi apparatus.[19] Transport of toxin to the Golgi

appears to be an important step in the intoxiation process as disruption of the Golgi with the drug brefeldin A or blocking Golgi transport with low temperatures (18 – 20° C) will prevent cell intoxication with STX.[18]

Ultimately the toxin reaches the endoplasmic reticulum (ER). The mechanisms of the transport are still unclear, although some have proposed that toxins in this family associate with or mimic the ER retrograde transport signal sequence, otherwise known as the KDEL amino acid sequence. Other proposals suggest that low numbers of lysine residues in these toxins allow these toxins to escape ubiquitin degradation in the ER.[20] During intracellular transport, the A subunit interacts with furin, a membrane bound enzyme in the endoplasmic reticulum. This enzyme cleaves the A subunit into the N-terminal A1 fragment (27 kDa) and a smaller 4 kDa A2 fragment. A disulfide bond initially joins the two fragments at cysteine 242 and cysteine 261, although eventually the catalytically active A1 fragment is released.[14] The A1 fragment acts to inhibit protein synthesis and causes cell death by removing adenine 4324 in the 28S RNA of the 60S ribosome.[13]

Red blood cell morphologic abnormalities are one of the classic features of HUS. Blood smears often reveal schistocytes, fragmented RBCs or spherocytes.[10,21] The mechanisms by which these changes develop remains unclear, however. The initial cause of RBC fragmentation was ascribed to membrane damage as cells flowed through fibrin thrombi, particularly those in renal vessels.[22] This mechanism was subsequently studied *in vitro* by observing RBCs flowing through a network of fibrin coated glass beads. The researchers observed hemolysis and RBC fragmentation in these cells that were subjected to adhesion and turbulent flow for 20 minutes.[23] Several researchers from that group also did

an *in vivo* study in rabbits that documented appearance of fragmented RBCs during and after induction of snake venom defibrination.[24]

Despite these findings, it has been puzzling that some patients with typical clinical signs and renal lesions of HUS lack the fragmented RBCs and hemolysis that was classically described.[25] One area of research on RBC abnormalities in HUS has focused on oxidative damage to red cells. The initial study in this area found RBCs from patients with HUS had decreased levels of phosphatidyl ethanolamine and plasma tocopherol. The authors of this study suggested that RBC oxidative damage may occur during HUS.[26] Their findings were subsequently confirmed by other researchers.[27] Other studies have added to the evidence of RBC lipid peroxidation by noting decreased levels of RBC arachidonic acid,[28] reduced glutathione[29] and superoxide dismutase[30] in HUS patients as compared to controls. A more recent study has again concluded that RBCs from HUS patients have evidence of lipid peroxidation and correlated this to lower red cell membrane fluidity.[31] These studies suggest that lipid peroxidation may contribute to the hemolysis observed in HUS patients.

Another possible mechanism for RBC damage and hemolysis is direct damage from *E. coli* toxins. In addition to shiga-like toxins, *E. coli* O157:H7 produces hemolytic toxins. The best known of these hemolytic toxins is the α-hemolysin, also known as HlyA. This toxin is a member of a group of similar toxins produced by a number of different bacteria. These toxins share tandemly repeated glycine-rich nonapeptides toward the carboxyl terminus of their structure that have given rise to the group name "repeats in toxin" (RTX).[32] HlyA, a product of the *hlyA* gene, is actively secreted from the cell utilizing a transport system composed of HlyB, HlyD and the outer membrane protein TolC.[33] To be fully functional, lysine residues 563 and 689 of HlyA must undergo acylation by HlyC.[34]

Morphologic studies have shown that hemolysin binding to sheep RBCs was sufficient to induce shape changes within five minutes of treatment.[35] More recent studies have shown that HlyA induces hemolysis by insertion of pores in the membrane that leads to influx of water and eventual bursting of the cell.[36] Binding of HlyA to the cell is independent of temperature and calcium concentration,[37] while lysis of the cell can only occur if calcium binds to the tandem repeat sequences prior to cellular adhesion.[38]

In addition to HlyA, another RTX family hemolytic toxin from *E. coli* has been recently described.[39] This toxin, termed "enterohemolysin" (Ehx) is encoded on the 90 kb plasmid pO157 and lyses fewer cell types than other RTX toxins.[40] Unlike HlyA, Ehx activity was not found in culture supernatants and suggests that Ehx is not actively secreted from the cell.[41] There appears to be some controversy in the literature concerning the substrate requirements and cell specificity of Ehx with one study reporting that Ehx was not calcium dependent and was unable to lyse human RBCs[41] and another observing a calcium requirement and the ability to lyse human RBCs.[40] One aspect of the newly identified toxin that is of particular interest is the strong correlation of human disease to the presence of Ehx. In bacterial isolates from patients with HUS, 16 of 18 were enterohemolytic and expressed Ehx, while only 4 of 18 isolates from other diarrhea cases were positive for Ehx.[42] In addition, 19 of 20 HUS patients had Ehx antibody in recovery serum samples, while only 1 of 20 controls had a similar antibody.[39]

While red cell fragmentation and hemolysis are important clinical features of HUS, the role of toxin binding to RBCs in the pathogenesis of the other clinical signs and histologic lesions has also received some research attention. One study specifically examined the ability of different toxins within the shiga toxin family to bind to RBCs of

various P blood groups. They found that binding of STX 1 and STX 2 to RBCs correlated to the amount of Gb3/P^k antigen expressed on RBCs; cells from donors that lacked this antigen failed to bind STX 1or STX2. The blood groups that express the P^k antigens are phenotypes P1 (also express P and P1 antigens), P2 (also expresses P antigen) and P^k2 (expresses only P^k antigen). This study also found that STX 2_e binding to RBCs correlated with expression of the Gb_4/P antigen.[43] Interestingly, the amount of toxin binding to RBCs may influence an individual's susceptibility to HUS. Two studies found that patients that developed HUS were likely to have lower levels of Gb_3 on their RBCs than children undergoing routine venipuncture[44] or patients with diarrhea caused by STEC that did not develop HUS.[45]

In addition to binding to RBCs, STX has been shown to bind to platelets. Gb_3 is expressed on platelets, RBCs, and endothelial cells.[46] A more recent study has found that STX binds to platelet Gb_3, and also binds to a newly discovered glycolipid, termed band 0.03. The presence of this glycolipid was related to high expression of Gb_3 and was thought to represent the structure IV^3-β-Galactose α[1→4] galactosylglobotetraosyl ceramide. Interestingly, this study also found that higher levels of Gb_3 expression did not correlate with increased binding of STX, but suggested that increased toxin binding occurred on older platelets.[47] Although platelets can bind toxin, it appears that STX 1 or STX 2 do not directly enhance aggregation of human adult platelets *in vitro*.[48]

The major contributor to the lesions and clinical signs in HUS is thought to arise as a result shiga toxin damage to endothelial cells (EC). Much of the original literature examining the effect of STX on EC were done on human umbilical vein EC, aortic EC or other cells easily attained or cultured. However, it has become clear that endothelial cells from different locations have different properties and respond to stimuli differently.[49,50]

Similarly, it has been found that endothelial cells from different locations respond to shiga toxin differently. Umbilical vein EC were found to be less sensitive to shiga toxin than renal microvascular EC. This sensitivity was found to correlate to the amount of Gb_3 found on these cells.[51] Another paper compared endothelial cells from different locations from neonates and adults and noted that neonatal cells were more sensitive to shiga toxin than adult cells and the sensitivity correlated to the amount of Gb_3 present on the cells.[52] Interestingly, confluence of cell cultures and stage of the cell cycle has been shown to influence susceptibility of vero cells to shiga toxin without alteration of the Gb_3 content.[53] It was shown that the maximum susceptibility to shiga toxin occurred around the G1/S transition.[54] A variety of chemical agents, cytokines and lipopolysaccharide have been studied in their ability to increase endothelial cell sensitivity to shiga toxins. Sodium butyrate has been found to increase the shiga toxin sensitivity of endothelial cells from some, but not all, locations.[55,56] Lipopolysaccharide has been found to enhance shiga toxin damage to endothelial cells in some studies,[57,58] while in other studies it did not promote enhanced endothelial cytotoxicity from shiga toxins.[59]

An important proposed mechanism in the development of HUS is an imbalance of the coagulation system. Thrombin generation appears to be increased during HUS as evidenced by increased levels of thrombin-antithrombin III complex.[60,61] Children with HUS have been found to have increased levels of fibrin degradation products and d-dimers, suggesting enhanced activity of the fibrinolytic pathway.[60,61,62] In addition, plasminogen activator inhibitor (PAI-1) levels were found to be significantly elevated in HUS patients.[60,62,63] During an *in vitro* study, it was found that renal endothelial cells responded to shiga toxin exposure more profoundly than umbilical endothelial cells by decreasing production of

urinary plasminogen activator (uPA). This decrease in uPA was suggested to promote overall reduction in renal fibrinolysis and promotion of renal thrombosis during HUS.[64] Recently, a serine protease produced by some *E. coli* O157:H7 isolates has been described. This protein, named EspP, has been shown to cleave human coagulation factor V.[16] It is unclear at this time how important EspP is in the pathogenesis of HUS.

Another component of the coagulation pathway that has been studied with respect to the pathogenesis of HUS is abnormalities of von Willebrand factor (vWF) release. von Willebrand factor is a multimeric protein that is produced exclusively by megakaryocytes and EC, where it is stored in granules called Weibel-Palade bodies. Its primary function is to stabilize platelet adhesion to subendothelium after vascular damage; it also prolongs the half-life of coagulation factor VIII in plasma. von Willebrand factor is found in the plasma in a variety of sizes, the largest of which are the most hemostatically active.[65] Most children with HUS have been found to have elevated levels of vWF in their plasma at the time of admission.[62,66] Examination of the vWF multimer sizes reveals that these patients usually have decreased amounts of the largest multimer sizes, although occasional patients have unusually large vWF multimers in their plasma.[66,67] It has been postulated that disappearance of the unusually large vWF from plasma is a result of consumption of those multimers in the thrombi that occur in the renal microvasculature.[67] Incubation of human umbilical vein EC with STX for 30 minutes was found to increase the amount of vWF and unusually large vWF in the culture supernatant.[68] In contrast to a similar clinical syndrome, thrombotic thrombocytopenic purpura (TTP), patients with HUS have normal levels of plasma vWF cleaving protease.[69]

In addition to effects on EC, the effect of shiga toxins on leukocytes may be important in the pathogenesis of HUS. Exposure to shiga toxin has been shown to enhance production of IL-1, TNF_{α} and other cytokines from human monocytes *in vitro*.[70] In addition to effects on monocytes, STX effects neutrophils as well. Patients with HUS have been found to have increased adhesion of neutrophils to EC as compared to controls.[71] An *in vitro* study also found enhanced adhesion of neutrophils to human umbilical vein EC after incubation with STX-1. This adhesion was blocked with antibodies to E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1.[72]

Thesis Organization

The objectives of this study were to determine a portion of the mechanisms underlying the fragmentation and damage that occurs to RBCs during HUS. This thesis will be divided into two papers that are to be submitted for publication. The first paper is entitled "Use of Computerized Image Analysis to Assess Bovine Erythrocyte Morphology" and was designed to examine the usefulness of a computerized image analysis in determining the amount of RBC fragmentation that occurred in an animal model of *E. coli* O157:H7 infection. The second paper is entitled "Direct and Interactive Effects of Shiga Toxin-1 on Red Blood Cells and Endothelial Cells." This study examined the direct effects of STX I on human RBCs using osmotic fragility and electrophoresis of RBC membrane proteins. In addition, this project examined the interactive effects of STX I on endothelial cells and RBCs. The final portion of this thesis is arranged as appendices and details the electrophoresis data obtained for the second publication. The second appendix shows the

results of a two-dimensional electrophoresis project on RBCs. The final appendix is the protocols used for the various experiments of this thesis.

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USE OF COMPUTERIZED IMAGE ANALYSIS TO ASSESS BOVINE ERYTHROCYTE MORPHOLOGY

A paper to be submitted to *Veterinary Clinical Pathology*

Denise Wunn, Claire Andreasen, Margaret Carter, Mark Ackermann, Evelyn Dean-Nystrom

Abstract

Marked poikilocytosis, including schistocytes and irregularly spiculated acanthocytes, were found in red blood cells (RBC) from calves experimentally infected with *Escherichia coli* O157:H7. A method was needed to improve evaluation of RBC morphology and to quantify the degree of poikilocytosis that occurred in 4 calves prior to infection and 10 calves after infection. The calves ranged from newborn to 4 months of age. Data was collected using a digital imaging system (Visilog, Noesis Vision, Inc). Area and crofton perimeter were measured and used to calculate a shape factor for each RBC. The shape factor was determined to be significantly different from 1.00 (perfectly round) at >1.24. The percentage of cells with a shape factor of >1.24 was compared between infected and control calves. For visual assessment, 1000 RBC per calf were evaluated independently and placed in one of seven shape categories. No significant differences were found between image analysis and manual scores, either prior to infection or after infection. No significant differences were found between age groups. Crenation of RBC caused the greatest discordance between the visual assessment and the image analysis scores. Samples with a higher percentage of crenation often resulted in a higher percentage of cells with a shape factor >1.24, rather than samples with 3+ or 4+ poikilocytosis (acanthocytes and schistocytes). The image analysis system was sensitive to the cumulative effects of shape changes, such as crenation, that may

occur as an artifact, and may not specifically detect diagnostically significant RBC shape changes, such as acanthocytes or schistocytes.

Introduction

The pathogenesis of *Escherichia coli* O157:H7 infection and associated hemolytic uremic syndrome (HUS), has been studied in calves and other animal models.[1] During our study of neonatal calves experimentally infected with *E. coli* O157:H7, it was noted that they had increased numbers of RBC shape changes such as acanthocytes or schistocytes when compared with normal calves. Although many pathogenic mechanisms of HUS have been studied extensively, the pathogenesis of the classically described RBC fragmentation remains unclear. The proposed mechanism is that the RBC membrane is damaged as they flow through platelet thrombi in the microvasculature.[2,3] It also has been proposed that RBC may be injured directly by bacterial toxins.[4]

The degree of poikilocytosis in blood smears has traditionally been graded on a subjective scale of 1+ to 4+.[5] Poikilocytes can also be quantified by manually counting a given number of RBC and noting the percentages of cells with a given morphologic abnormality. However, these methods can be subjective and time consuming. Computerized image analysis offers a means of detecting and more objectively quantifying shape changes that might occur in RBC. Image analysis of RBC shapes has been described in several human medical publications, particularly in the area of sickle cell anemia.[6,7,8,9,10,]

Although several different shape, size and color features of RBC can be measured using image analysis, one report found that a single "form factor" equation ($\{4 \bullet \pi \bullet \text{Area}\} \div \{\text{Perimeter}^2\}$) was the sole factor needed to classify RBC as normal, abnormal

or sickle.[11] To our knowledge, there have been no reports utilizing image analysis in the study of RBC shapes in domestic animals. The objective of this report is to compare, using both manual methods and computerized image analysis with a single shape factor equation, the numbers of poikilocytes in calves prior to and after infection with *E. coli* O157:H7.

Materials and Methods

Samples

Samples were taken from 4 calves prior to infection and 10 calves after infection with *E. coli* O157:H7. Six of these were paired samples from colostrum-deprived neonates (less than 3 days of age); the remaining samples were taken from calves 4 months of age. Two neonatal calves were infected with a non-pathogenic *E. coli* to serve as a control. The remaining neonatal calves were infected with *E. coli* O157:H7, strain 933. Four-month-old calves that had been fasted 48 hours were inoculated orally with 10^{10} colony forming units of *E. coli* O157:H7 strain 5570. Samples were collected from calves at 2 days post infection (neonates) or at 4 days post infection (weanlings) immediately prior to euthanasia and necropsy. Blood was collected into potassium EDTA and analyzed within 3 hours of collection. Complete blood counts with plasma protein, fibrinogen and manual differentials were performed on all calves. Blood smears were routinely prepared and stained with Wright's stain.[12]

Image Analysis Parameters

The slides were examined using 1000X magnification on a microscope fitted with a color video camera and attached computer hardware.[13] Images from 10 randomly selected

fields in the monolayer were captured using image analysis software (Visilog by Noesis Vision, Inc.) with a resolution limit of approximately 0.21 microns per pixel. Leukocytes and platelets were excluded from the image based on their blue staining characteristics. Images of incomplete RBC on the edge of the field also were discarded. The mean number of RBC analyzed was 596 per sample with the minimum number 368 and the maximum being 747 RBC per sample. Area and crofton perimeter were measured for each cell. Area is a geometric measurement that is determined by the number of pixels contained within given boundaries of an object. Crofton's perimeter formula is a means of calculating perimeter based on the lengths of lines that intercept the edge of a given object. A "shape factor" (SF) was calculated for each cell using the formula $SF=(\text{crofton perimeter}^2) \div (4 \bullet \pi \bullet \text{area})$.[14] Perfectly round objects have a SF of 1.00; linear objects have a SF approaching 2.00. Previous analysis of three clinically healthy calves found that normally shaped RBC had a SF of less than 1.25. The percentage of RBC with SF equal or greater than 1.25 was determined for each calf.

Manual Scoring of RBC Shapes

Two investigators (DW & CA) independently scored the number and type of poikilocytes from each calf. 1000 RBC were counted and each RBC assigned into one of 7 categories: discocyte, crenated, schistocyte, acanthocyte, keratocyte, dacryocyte and irregular poikilocyte.[15] The total number of poikilocytes from calves prior to infection with *E. coli* were compared to the number of poikilocytes after infection. Manual scores also were compared to the numbers of poikilocytes from image analysis.

Statistical Analysis

Data was analyzed using a Student's T-test for parametric data or a Wilcoxon's Rank Sum Test for non-parametric data. P values less than 0.05 were considered significant.

Results

Hematology

Neonatal calves had significantly higher plasma fibrinogen after infection as compared to before infection ($p = 0.003$, Fig 1). Calves infected with *E. coli* O157:H7 had significantly lower numbers of total white blood cells and mature neutrophils after infection as compared to before infection ($p = 0.002$ and $p = 0.0001$); however, calves receiving the non-pathogenic strain of *E. coli* also had significantly lower numbers of total leukocytes and mature neutrophils. The remaining hematologic data was not significantly different after infection as compared to before infection (Fig 2).

Image Analysis and Manual Scoring

The percentage of poikilocytes from image analysis was not significantly different in calves prior to infection with *E. coli* O157:H7 as compared to post infection ($p = 0.39$, Fig 3). Although there was a trend toward higher numbers of crenated RBC, schistocytes, and acanthocytes after infection, the large amount of variation between calves did not allow for a statistical significance. There were no statistically significant differences in image analysis scores between neonatal and 4 month old calves. There were no statistically significant differences in any manually scored shape category prior to infection as compared to post infection (Fig 4). There were also no differences in manual RBC scores between the

neonatal and weaned groups. The samples with the highest percentage of poikilocytes given by image analysis scores had the highest numbers of crenated cells by manual scoring methods. Shape factor did not correlate well with any one shape category, although the best correlation was with crenated cells ($r = 0.65$).

Discussion

The significantly increased fibrinogen and decreased number of total white blood cells and mature neutrophils after *E. coli* O157:H7 infection are consistent with acute inflammation and increased tissue demand for neutrophils. These are common findings in calves with diarrhea due to a number of different etiologic causes and should not be considered specific for *E. coli* O157:H7. While the neonatal calves developed colitis, they did not develop other signs or lesions consistent with HUS. It should be noted however, that HUS in children usually occurs 2 to 14 days after the onset of diarrhea.[16] It is possible that in euthanizing these calves on days 2 or 4 post infection, the classic hemolytic uremic syndrome was not allowed time to develop.

While there was a higher mean shape factor score from image analysis in calves after infection with *E. coli* O157:H7, the trend was not statistically significant. It is also noted that calves tended to have higher numbers of crenated RBC after infection (although not statistically significant). The reason for the apparent increase in crenation is not clear, although it is possible that switching of hemoglobin types during the neonatal through weaning periods has influenced these results. Previous reports have noted that younger calves are more likely to have poikilocytosis than those greater than six weeks of age.[17] Calves have embryonic, fetal and adult types of hemoglobin at birth and gradually change to

production of only adult hemoglobin by 6 to 7 months of age.[18] In cattle, the switch from fetal to adult hemoglobin is associated with a structural change in the red cell that is correlated with a decrease in mean corpuscular volume.[19] Other reports have proposed that iron deficiency or abnormalities in hemoglobin expression may account for the high number of poikilocytes in calves.[20]

Our goal in this study was to evaluate computerized image analysis and a single shape factor calculation as an objective, quantitative method of detecting schistocytes and fragmented RBC in blood smears from calves infected with *E. coli* O157:H7. In our study, the shape factor correlated most strongly with crenated RBC. Use of image analysis in species and age groups that tend to have crenated RBC may lower the sensitivity of detecting more diagnostic shape changes, such as schistocytes. Image analysis and single shape factor calculation of RBC may be more useful in other species or in mature animals less prone to artifactual crenation of their RBC. Perhaps the relative resistance of human RBC to crenation allowed previous investigators to use a single shape factor calculation to successfully identify sickled red cells.[11] Other researchers have determined up to ten shape and color parameters for each red cell and devised elaborate graphs and tables to arrive at an erythrocyte differential using a computerized image analysis system.[8] In the future, use of additional image analysis shape parameters might prove helpful in identifying schistocytes or fragmented RBC and differentiating them from crenated cells in veterinary species.

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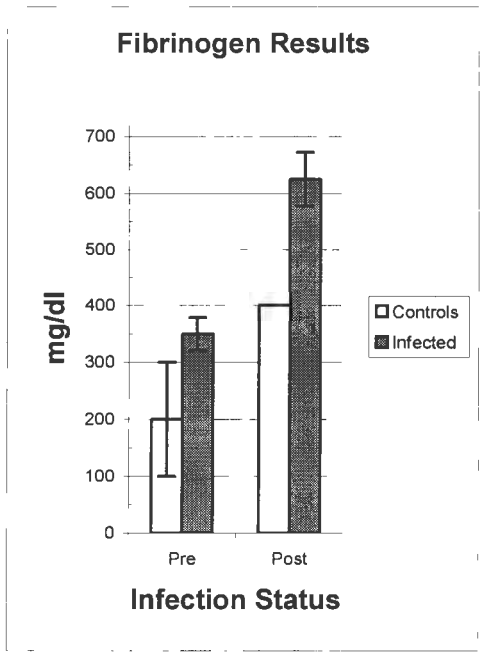


Fig 1—Mean fibrinogen levels in neonatal calves (n=2) prior to and after infection with a non-pathogenic strain of *E. coli* (white bars). Standard error of post infection control calves is zero. Grey bars show fibrinogen levels in neonatal calves (n=4) prior to and after infection with *E. coli* O157:H7. Significantly different from controls at $p = 0.003$.

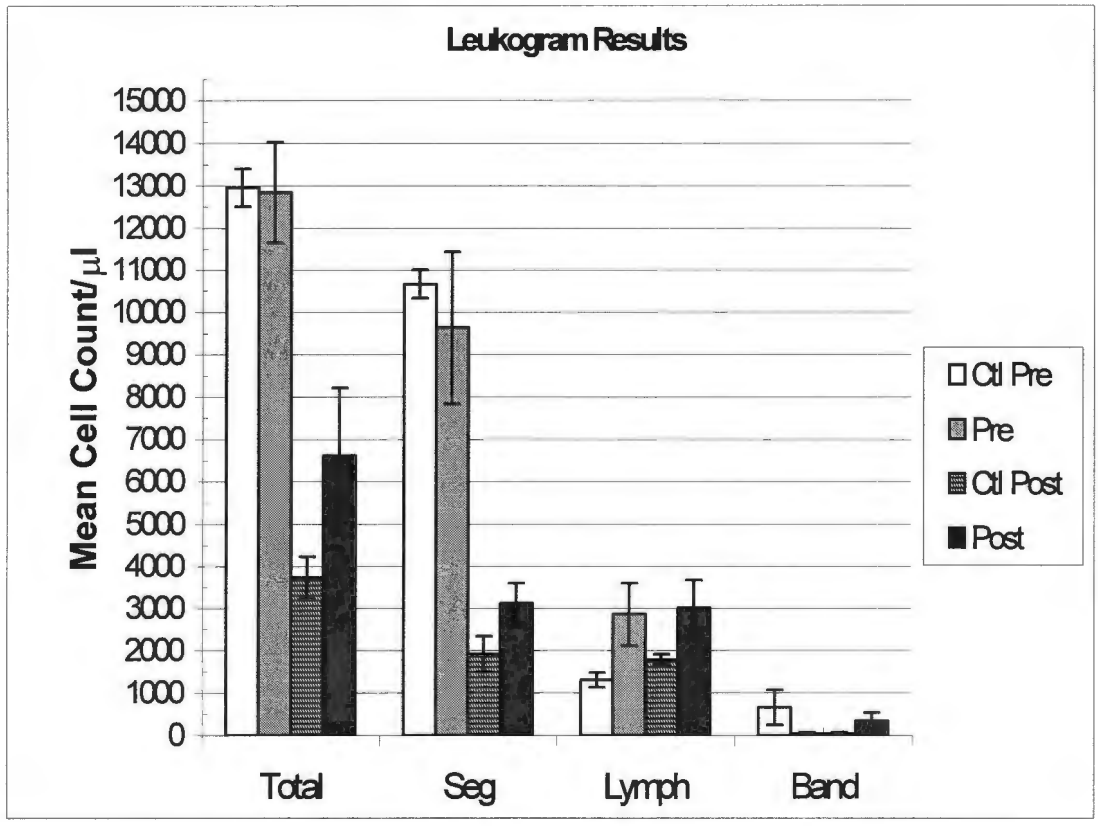


Fig 2—Leukogram data showing total mean white blood cells, segmented/mature neutrophils, lymphocytes and band/immature neutrophils. White bars are from neonatal calves (n=2) prior to infection with a non-pathogenic strain of *E. coli.* Dashed bars are after infection with non-pathogenic *E. coli.* Grey bars are from neonatal calves (n=4) prior to infection with *E. coli* O157:H7. Black bars show calves after infection with *E. coli* O157:H7. Total WBC and segmented neutrophils were significantly different in neonatal calves after infection with *E. coli* O157:H7, however, neonatal calves with non-pathogenic *E. coli* were also significantly different.

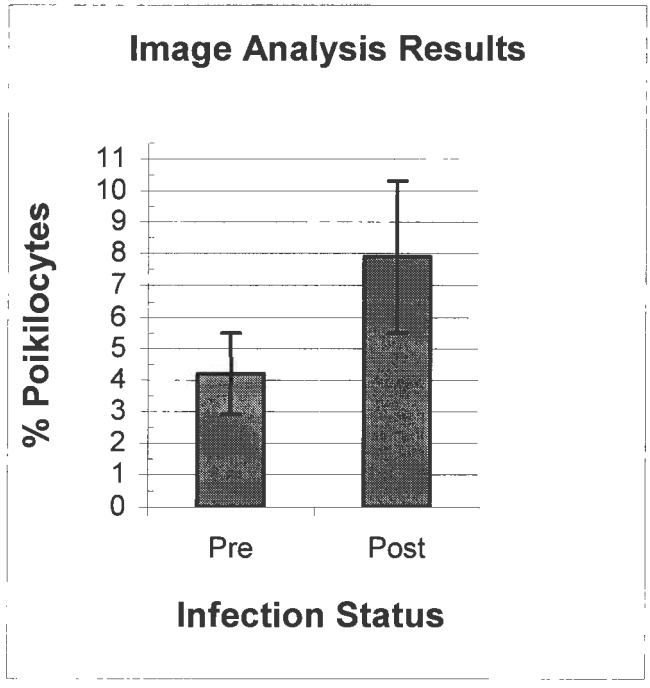
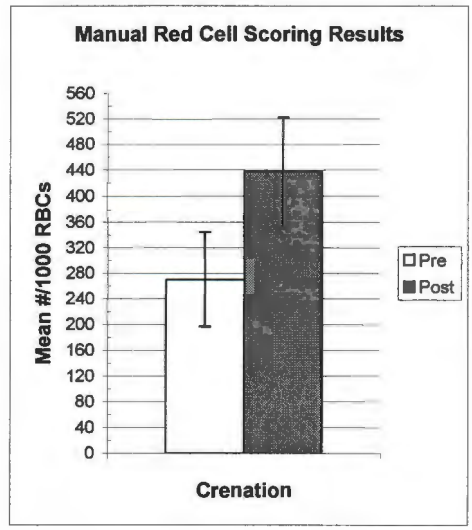


Fig 3—Percentage of poikilocytes as given by computerized image analysis in calves pre-infection (n=4) and post-infection (n=10) with *E. coli* O157:H7. Results not statistically significant ($p = 0.46$).



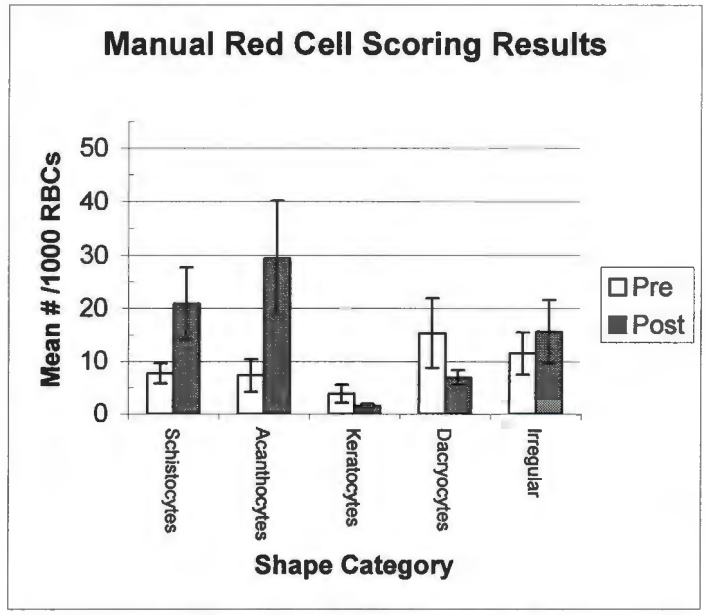


Fig 4—Mean numbers of crenated RBC (top) and other shape changes (bottom) given by manual scoring in calves prior to and after infection with *E. coli* O157:H7. No results were statistically significant at the p=0.05 level.

DIRECT AND INTERACTIVE EFFECTS OF SHIGA TOXIN-I ON RED BLOOD CELLS AND ENDOTHELIAL CELLS

A paper to be submitted to *Journal of Infectious Diseases*

Denise Wunn, Claire Andreasen, Ingrid Pruimboom, Brad Fenwick

Abstract

Fragmented red blood cells (RBCs) are one of the hallmark features of hemolytic uremic syndrome, a disease that develops after infection with *Escherichia coli* O157:H7 and exposure to its Shiga-like toxin (STX). This study examined the direct effects of STX on RBCs and their interaction with cultured endothelial cells. Human RBCs were incubated with STX for 30 and 60 mins and 24 hrs. No significant changes were seen in RBC morphology as determined by light microscopy or osmotic fragility. Using a gravity adherence assay, no increase in RBC adhesion to STX incubated endothelial cells was observed. Direct exposure to STX did not alter the RBC membrane proteins visualized using gel electrophoresis. Similarly, RBCs incubated with STX exposed endothelial cells did not have altered membrane proteins. The mechanism of RBC fragmentation is likely the result of indirect effects of STX or combinations of toxins produced by *E. coli* O157:H7.

Introduction

Escherichia coli O157:H7 infects more than 73,000 people each year and causes approximately 65 deaths; mostly in children that develop hemolytic uremic syndrome (HUS).[1] In 1955, Conrad von Gasser reported the first cases of HUS and described the hallmark features of fragmented red blood cells (RBCs), acute renal failure and

thrombocytopenia.[2] The development of HUS has since been associated with infection by *E. coli* O157:H7 and the Shiga-like toxin(STX) it produces.[3]

The fragmentation of RBCs has been attributed to damage caused during circulation past microvascular thrombi that are formed in response to STX exposure. However, previous studies of RBC shape changes in sheep RBCs noted that attachment of an *E. coli* α-hemolysin induced rapid shape changes followed by hemolysis.[4] In other diseases with RBC fragmentation (sickle cell anemia), RBCs have been shown to adhere to cultured endothelial cells.[5,6] To date, there have been no studies examining the direct effects of STX on the morphology of RBCs. Further, there have been no studies exploring the adhesion of RBCs to endothelial cells after STX exposure. The objective of this study was to test for direct effect of STX on RBCs by measuring the degree of RBC fragmentation and membrane protein alteration. In addition, the interactive effects of RBCs and endothelial cells were studied.

Materials and Methods

Direct effects of STX on RBC morphology.

Whole blood was collected from a human volunteer into potassium EDTA and lithium heparin tubes for duplicate trials. The samples were divided into two with one sample being retained as whole blood and the cells in the remaining sample prepared as washed RBCs. Washed RBCs were prepared by centrifuging; discarding the plasma and washing the RBCs three times with phosphate buffered saline (PBS). Washed RBCs were resuspended in PBS to a concentration of ~35%. Two μg Shiga-like toxin I (STX, provided by Dr. Brad Fenwick, Kansas State University) were added to tubes containing 250 μl of whole blood or washed RBCs and were incubated at 37°C for 30 and 60 minutes and 24

hours. After incubation, blood smears were made, stained with Wright's Giemsa and observed microscopically for changes in erythrocyte morphology as compared to smears made prior to incubation.

RBC osmotic fragility.

The osmotic fragility assay was done using the method described by Schalm.[7] A 1% solution of saline (pH 7.4) was diluted in 15 tubes with incrementally increasing amounts of distilled water resulting in 0.85% to 0.10% NaCl solutions. A final tube contained only distilled water. Ten µl of either whole blood or washed RBCs incubated with STX at 37°C for 30 and 60 minutes and 24 hours were added to each tube in the series and incubated at 22°C for 30 minutes. A sample of blood incubated at 37°C for the same time without STX served as a negative control. After incubation, the tubes were centrifuged and the spectrophotometeric absorbance of the supernatant from each tube was recorded at 540 nm. The results were plotted as percent hemolysis per concentration of saline. The concentration at which 50% hemolysis occurred was determined using probit analysis. This experiment was performed twice.

STX effects on endothelial cells.

The effect of STX on endothelial cells from different locations was studied on commercially obtained (Clonetics, Inc., Walkersville, MD) human umbilical vein endothelial cells, human renal arterial and human dermal microvascular endothelial cells during three replicate trials. Confluent monolayers of endothelial cells were incubated for 24 hours in chamber slides. The chambers contained: 1.) culture media (EGM-2MV®, Clonetics, Inc., Walkersville, MD) and 1 µg STX, 2.) 1 µg STX; and 10 µg E. coli lipopolysaccharide (LPS) (Sigma Chemical Co., St. Louis, MO) in culture media, 3.) 10 µg LPS in culture media and

4.) media only (negative control). Cytotoxicity was subjectively evaluated by observing the numbers of detached cells. Binding of STX was confirmed using immunofluorescence. After incubation with culture media or toxin, cells were washed, the chamber removed and incubated with polyclonal mouse anti STX-1 antibody (provided by Dr. James Samuel, Texas A&M University) for 30 minutes at 37°C. The cells were then washed, fixed in 4°C methanol and air dried. They were then incubated with fluorescein labeled mouse anti-IgG for 2 hours in the dark at 22°C. The slides were covered with glycerol mounting media that contained propidium iodide (VectaShield®, Vector Laboratories, Burlingame, CA) and viewed with ultraviolet microscopy.

RBC/endothelial cell adhesion assay.

The method of determining RBC/endothelial adherence was adapted from a published assay[6] and replicated four times. Human dermal microvascular endothelial cells from neonates were grown to confluence on chamber slides. Cell monolayers were exposed to media that contained 1 μg STX or 1 μg STX and 10 μg LPS for 24 hours at 37°C. After incubation, media containing STX was removed and 1.5 ml whole blood or washed RBCs (see above) were added to the chamber and the slide rocked for 30 minutes at 37°C. The chamber was then filled with PBS and inverted for 20 minutes at 22°C. The chamber was removed; the slide dipped once in PBS, air-dried and the cells stained with a modified Wright's stain. The slide was observed microscopically and the numbers of adherent RBCs noted.

RBC membrane protein electrophoresis.

RBC membrane proteins were analyzed using the method described by Barker.[8] Five mls of whole blood was collected for four replicate experiments from a volunteer into lithium

heparin, and divided into three samples. One 2.5 ml aliquot was maintained as a control, while the other aliquots were incubated with 1 μg STX for 1 and 3 hours at 37°C. A separate RBC sample was incubated for 3 hours with human microvascular endothelial cells that had been exposed to STX for the previous 24 hours. All RBC samples were then processed for RBC membrane protein electrophoresis. The whole blood was centrifuged; the plasma and buffy coat removed and the RBCs washed 6 times with PBS (pH 7.4), removing residual buffy coat after each wash. The cells were lysed with 50 mls of 20 mM Tris (pH 7.6) at 4°C and centrifuged at 30,000 g at 4°C for 30 minutes. The supernatant was removed and the membranes washed and centrifuged until the supernatant was clear. Residual leukocytes and platelets appeared as white pellets and were removed with cotton-tipped applicators. After preparation, RBC membranes were resuspended in the Tris solution, standardized by measuring absorbance at 540 nm, aliquoted and frozen at -70°C until use. RBC membranes were suspended in equal volumes of sample buffer (0.0625 M Tris-HCl, pH 6.8, 8M urea, 5% SDS, 5% 2-mercaptoethanol, 10% glycerol & 0.0125% bromophenol blue). Electrophoresis was carried out using 10 well, 12% polyacrylamide 10 X 10 cm gels in Tris/glycine/SDS buffer (Bio-Rad Co., Hercules, CA). Gels were run at 100 V for 1 hour 20 minutes. A standard protein solution was used in every gel (high molecular weight standard mixture, Sigma Chemical Co., St. Louis, MO). Gels were stained using 0.5% Coomassie Brilliant Blue, 45% methanol and 10% glacial acetic acid for 1 hour at 22°C. They were then destained using several changes of 45% methanol and 10% acetic acid solution and constant gentle agitation. Gels were rinsed with water and photographed using a gel imaging computer program (GelExpert®, Nucleotech, San Mateo, CA).

Results

Direct effects of STX on RBC morphology.

After incubation with STX, RBCs from whole blood and washed RBCs did not exhibit increased fragmentation or poikilocytosis as compared to whole blood and washed RBCs not incubated with toxin.

RBC osmotic fragility.

There was no change between cells that had been exposed to STX as compared to negative controls (Fig 1). These findings are similar to clinical studies that observe no change in RBC osmotic fragility in HUS patients.[9]

STX effects on endothelial cells.

Human umbilical vein endothelial cells showed no cytotoxic effects (few detached cells) after incubation with 1 pg to 3 mg STX for 24 hours. Similarly, renal arterial endothelial cells exhibited no cytotoxic effects to 1 μg STX after 24 hours. Despite no cytotoxic effects, positive immunofluorescence deposition demonstrated that STX had attached to these cells. Human microvascular endothelial cells were strongly sensitive to the cytotoxic effects of STX and had subjectively increased amounts of immunofluorescence. Cells that had been incubated with STX exhibited ~75% cell death as compared to negative controls. Cells incubated with LPS alone did not demonstrate immunofluorescence.

RBC/endothelial cell adhesion assay.

After incubation, the numbers of RBCs adherent to endothelial cells were not significantly different from negative controls ($p=0.68$, Fig 2).

RBC membrane protein electrophoresis.

Electrophoresis revealed RBC membrane proteins were similar in type and concentration after exposure to STX for 1 and 3 hours (Fig 3). RBC membrane proteins that were exposed to STX-incubated endothelial cells were not different from control RBCs (Fig 4).

Discussion

E. coli Shiga-like toxin 1 was not found to directly induce RBC fragmentation or changes in RBC membrane proteins. Similarly, RBCs incubated with STX exposed endothelial cells demonstrated no changes in their membrane proteins using SDS-PAGE. Other researchers have studied the direct effects of STX on cells other than RBCs. One recent study indicated Shiga toxins 1 and 2 do not directly enhance platelet aggregation.[10] There also was no change in RBC osmotic fragility after exposure to STX. This is in contrast to the α-hemolytic toxins that have been shown to insert into the membranes of RBCs and form pores that allow leakage of intracellular ions. This contributes to RBC morphologic changes[5] and eventual RBC rupture due to colloidal osmotic shock.[11] While fragmented RBCs and thrombocytopenia have been described as two hallmark features of HUS, STX, the toxin most associated with development of the disease appears to contribute to these findings through indirect means.

This study fails to detect any direct effect of STX on RBCs. Perhaps this is attributable to the mature RBC lacking much of the cellular organelles that are needed for processing and transport of the toxin. Shiga toxins bind to glycolipid receptors (globotriaosyl

ceramide, Gb_3 or globotetraosyl ceramide Gb_4) on the cell surface and are transported to the endosomes, golgi apparatus and eventually the endoplasmic reticulum. One report stated that disruption of the golgi apparatus or blocking of toxin entry into the golgi by low temperatures prevents Shiga intoxication of cells.[12] Lacking golgi apparatus may be an important protective mechanism for the mature RBC against intoxication with STX. RBCs have been shown to bind STX to Gb3 receptors on their surface. Interestingly, higher concentrations of STX binding to RBCs have been theorized to decrease the susceptibility of children to develop HUS.[13]

As reported in prior publications, endothelial cells from different locations respond to STX quite differently.[14] Our study confirms that microvascular endothelial cells are more sensitive to STX than cells from larger caliber vessels. Previous reports indicate that the degree of sensitivity to STX is related to the amount of toxin binding.[15] Although not quantiatively studied, our results using an immunofluorescence assay parallel the published literature in noting that microvascular endothelial cells had subjectively increased toxin binding as compared to those cells from a large caliber location.

Despite evidence of RBC/endothelial cell adhesion in other diseases, we report no enhanced RBC adhesion after endothelial cell exposure to STX. One possible explanation for these findings is that our endothelial cells had been incubated with STX for 24 hours and approximately 50% of the cells had become detached from the slide. Also, removal of the media from the STX incubated cells prior to addition of RBCs may also have removed any adhesive proteins that may have been released by the endothelial cells into the media. One study found that von Willebrand factor release from sensitive endothelial cells correlated to the degree of cytotoxicity as soon as three hours after exposure to STX.[14] von Willebrand

factor has been shown to play an important role in the adhesion of RBCs to endothelial cells during diseases such as sickle cell anemia.[5,6]

Although RBC fragmentation and hemolysis has been described since the first case reports of hemolytic uremic syndrome, there is relatively little published on the mechanisms of these changes. This study demonstrates that STX is likely not the direct cause of RBC fragmentation, or RBC membrane protein changes during hemolytic uremic syndrome. Additionally, we found no evidence of RBC adhesion to endothelial cells after they had been exposed to STX. Continued studies are needed to define the mechanisms of RBC fragmentation and their interaction with endothelial cells during the course of infection with *Escherichia coli* O157:H7.

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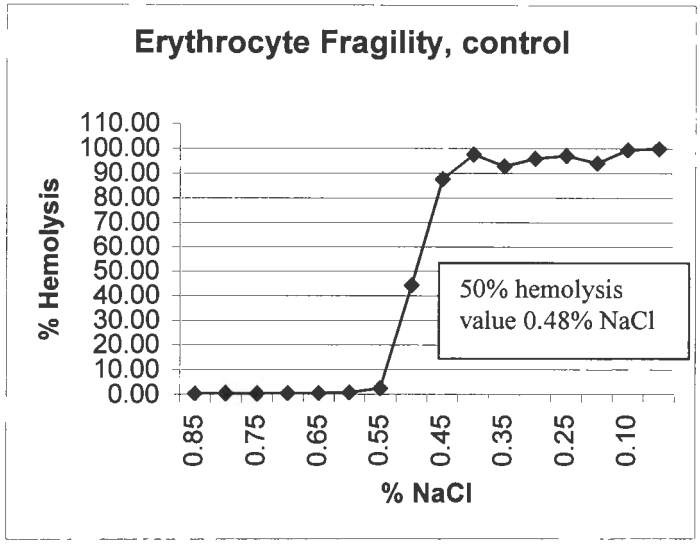

Figure 1a-Erythrocyte fragility of human whole blood prior to incubation with toxins n=2

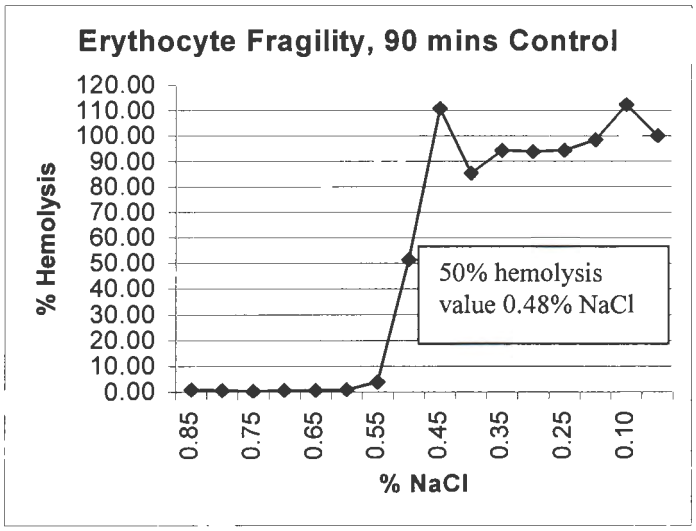

Figure 1b-Erythrocyte fragility after 90 mins incubation with saline

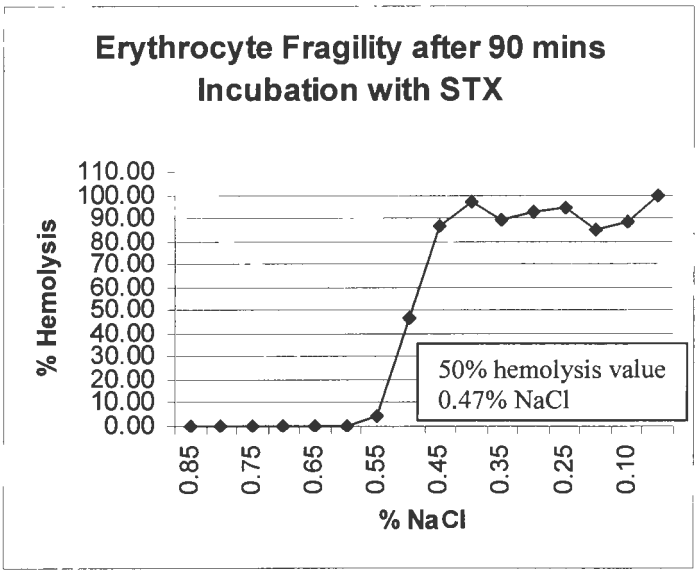

Figure 1c-Erythrocyte fragility after 90 mins incubation with STX

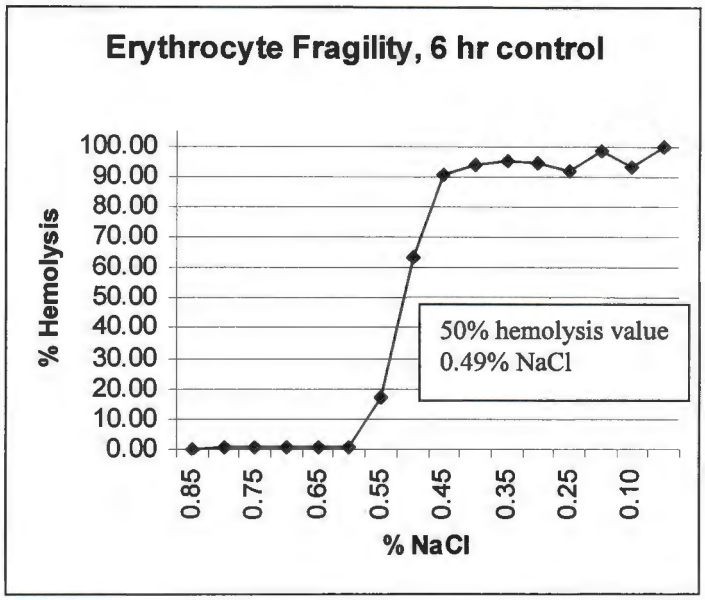

Figure 1d-Erythrocyte fragility after 6 hr incubation with saline

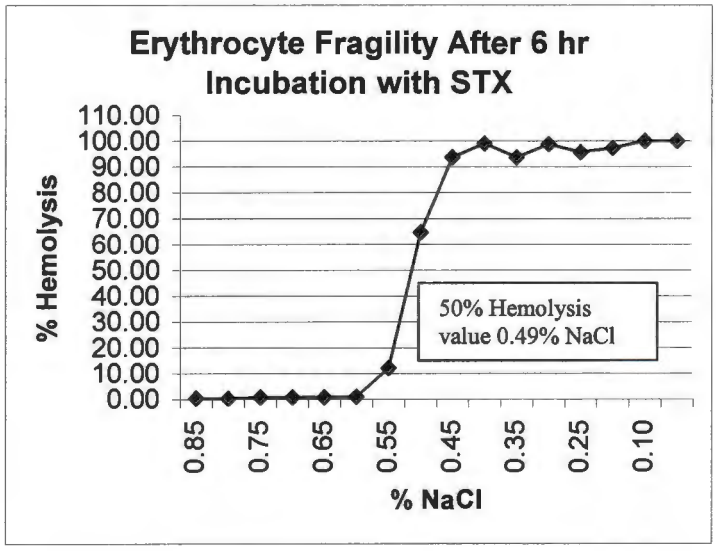

Figure 1e-Erythrocyte fragility after 6 hr incubation with STX

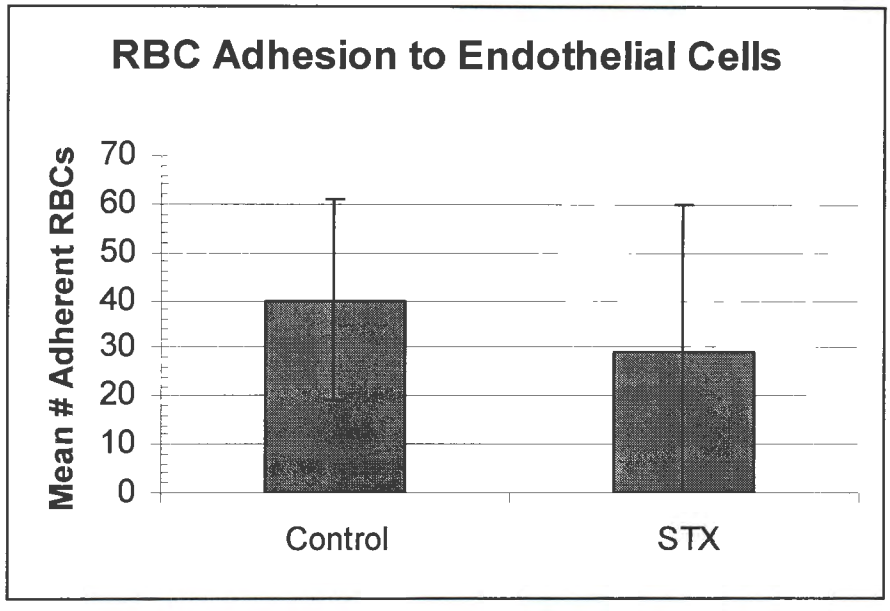

Figure 2-Data from RBC/endothelial adhesion assay showing mean numbers of adherent RBCs per 500x field. Control endothelial cells were incubated with saline for 24 hours, treated endothelial cells were incubated with STX for 24 hours. Results are not statistically significant ($p=0.68$).

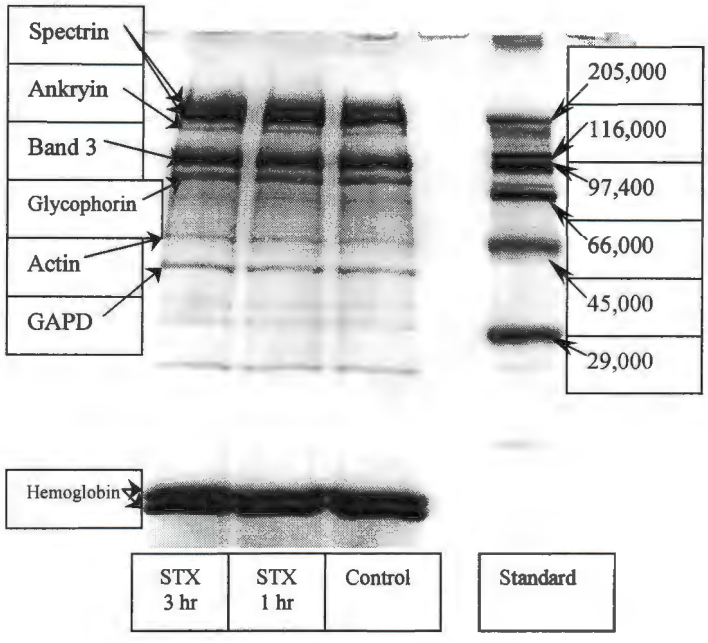

Figure 3- Electrophoresis of RBC membrane proteins after incubation with STX for different times (left 2 lanes) and no STX ("control"), standard protein mixture in right lane

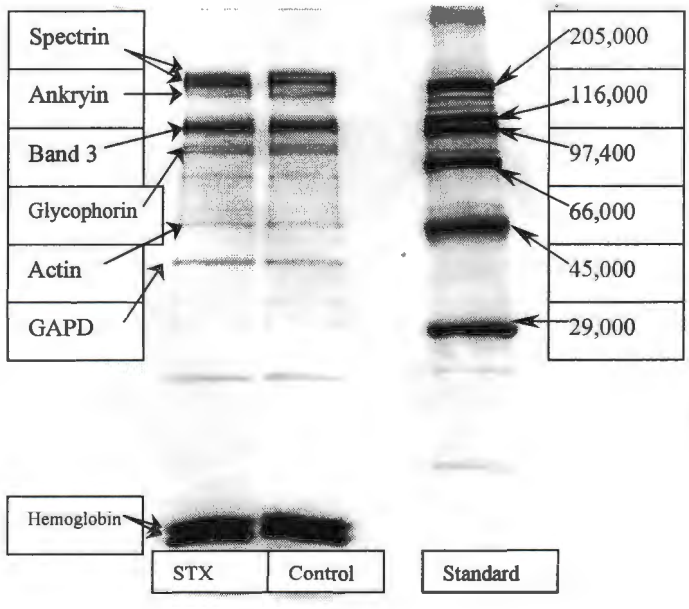

Figure 4- Electrophoresis of RBC membrane proteins after incubation with STX exposed endothelial cells (left lane) and after incubation with confluent endothelial cells (center lane). Standard protein mixture in right lane.

GENERAL CONCLUSIONS

Discussion

Previous work in humans with sickle cell anemia found that a single shape factor was sufficient to identify sickled RBCs.[1] In our studies, image analysis with single shape factor calculation was inadequate to separate fragmented RBCs from those that were crenated. Perhaps in future studies, additional shape factor parameters could be used to assist separation of schistocytes from crenated cells. Other image analysis parameters that may be useful include measures of RBC spicularity or elongation. Other studies using image analysis of human RBCs have used a number of image analysis parameters and calculations, often combined with various charts and graphs to classify RBC abnormalities.[2,3] The potential difficulty with using additional analysis parameters is the amount of time to process the data and perform additional calculations.

In order to quantify the amount of RBC fragmentation, computerized image analysis with a single shape factor equation was used to compare RBCs in calves before and after infection with *E. coli* O157:H7. Crenation is a particular problem in the calf, likely a result of switching from fetal hemoglobin to adult hemoglobin that occurs during the neonatal period.[4] Use of image analysis in this species during the neonatal period may prove unrewarding. Studies using image analysis of RBCs from other veterinary species with more stable RBC shapes, such as the dog, may be more fruitful. In addition, while the calf model may be useful for studying intestinal colonization, it is unlikely that the calf will be a useful model for the systemic effects of *E. coli* O157:H7 infections.

Calves infected with *E. coli* O157:H7 did demonstrate significant changes in other components of the complete blood count. The numbers of total leukocytes and mature

neutrophils was significantly decreased during infection with *E. coli* O157:H7. In addition, the fibrinogen level in plasma was significantly increased. These findings are consistent with inflammation and enhanced tissue demand for neutrophils. While these findings are helpful in further understanding how the calf responds to infection with *E. coli* O157:H7, hyperfibrinogenemia and neutropenia are common findings in a variety of bovine inflammatory diseases and not specific for *E. coli* colitis.

Direct effects of STX on RBCs have not been reported in the literature. Our studies found that incubation of human RBCs with STX does not cause any change in the RBC morphology or osmotic fragility after 30 minutes, 60 minutes or 24 hours. Unlike hemolysins, STX does not form pores in RBC membranes. The mechanism of intoxication by STX involves cellular uptake and eventual transport of the toxin to the golgi apparatus and the endoplasmic reticulum. STX damages cells by inhibiting protein synthesis.[5] Previous studies have shown that disruption of the golgi apparatus with the drug brefeldin A will prevent cellular intoxication with STX.[6] Given that mature RBCs lack cellular organelles such as golgi or the endoplasmic reticulum, it is not surprising that these cells are resistant to the direct effects of STX. Future studies on the pathogenesis of RBC fragmentation could focus on the effects of toxins HlyA or Ehx that insert pores into RBC membranes and do not rely on organelles that RBCs lack.[7]

The interactive effects of STX on EC and RBC were studied using a gravity adherence assay. While performing this assay, it was observed that umbilical vein EC and even renal arterial EC were much more resistant to the effects of STX I than were microvascular EC. These findings are similar to those previously published.[8,9] After EC incubation with STX, there was no increase in RBC binding to cultured cell monolayers.

These results were disappointing as RBC adherence to EC has been shown in several reports to be involved in the pathogenesis of vaso-occlusive events of sickle cell anemia patients.[10,11] Interestingly, one proposed mechanism of these adhesive events between sickle RBCs and endothelium was shown to involve unusually large multimers of vWF.[12] Differences in our study and the former that may bear consideration for future research is the use of different adhesion assays. Our study utilized a gravity flow assay while several studies in this area used a physiologic flow chamber to study RBC/EC adhesion. In addition, several sickle cell anemia studies used cells from sickle cell patients, many of whom had a reticulocytosis at the time of sampling. Our study utilized blood from healthy human volunteers with no evidence of reticulocytosis.

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APPENDIX A:

RBC MEMBRANE PROTEIN ONE-DIMENSIONAL ELECTROPHORESIS DATA

RBC Membrane Gel, 6-17-99

	Control		STX 1 hr		STX 3 hr	
Band	% Area	Position	% Area	Position	% Area	Position
1	12.68	59	16.65	67	22.07	68
2	15.73	67	15.70	74	2.56	77
3	7.31	82	1.32	85	4.52	85
4	8.81	105	15.42	106	14.37	104
5	6.61	119	6.76	121	6.18	118
6	3.43	134	3.72	137	3.21	134
7	6.26	166	6.37	168	6.53	166
8	9.62	191	6.35	192	5.98	190
9	2.92	220	4.82	221	4.57	220
10	1.90	236	1.04	238	2.80	236
11	3.28	274	4.39	274	4.47	274
12	21.45	383	17.44	382	22.73	385

RBC Membrane Gel, 6-15-99

	Control		STX 1 hr		STX 3 hr	
Band	% Area	Position	% Area	Position	% Area	Position
1	16.11	77	18.80	76	13.95	71
2	14.20	84	14.00	84	10.32	81
3	3.27	94	3.01	93	1.76	91
4	13.69	119	15.62	113	11.68	116
5	4.92	131	0.49	122	5.25	128
6	2.61	146	1.72	141	3.82	143
7	3.97	177	4.27	175	3.35	174
8	4.14	200	3.54	198	3.78	196
9	3.24	229	3.01	226	3.63	224
10	3.38	241	2.72	240	3.19	239
11	5.58	277	6.02	276	5.44	274
12	24.90	377	22.82	377	33.84	379

APPENDIX B:

TWO-DIMENSIONAL ELECTROPHORESIS OF RED BLOOD CELLS

In order to more fully examine the effects of Shiga-like toxin 1 (STX) on red blood cell (RBC) proteins, a two-dimensional electrophoresis (2D ELP) study was carried out. RBC membrane proteins as previously prepared (see RBC membrane protein electrophoresis protocol) and whole RBCs were examined using 2D ELP. A 2D ELP web site (http://www.expasy.ch/) sponsored by the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics was found and offered a great deal of information about 2D ELP including references, photographs etc. In addition, protocols from the Electrophoresis Laboratory at the Geneva University Hospitals can be found at: http://www.expasy.ch/ch2d/protocols/

The whole blood protocols were adapted from methods published by Golaz.[1,2] Blood was drawn from healthy human volunteers after obtaining informed consent. 5 mls whole blood was drawn into EDTA and centrifuged at 3500 rpm for 10 mins. The plasma and buffy coat was removed with a transfer pipette. The remaining RBCs were washed 4 times with phosphate buffered saline (pH 7.4). Care was taken to remove any residual buffy coat after each wash. Seven µl of packed RBCs were then added to 483 µl rehydration buffer. 5 µl of this solution was then added to 120 µl of rehydration buffer and the entire amount then added to the first dimension separation gel. The first dimension gels used were Immobiline Dry strips pH 3 – 10 linear, produced by Amersham Pharmacia. Some of our biggest problems were in using the proper amount of protein so as not to overload the gel and yet still get good

protein visualization. After several hours of rehydration and separation in the first dimension, the gels were removed and the placed in SDS equilibration buffer in order to quench the charge and allow separation based on molecular weight. If the gel was not to be run in the second dimension immediately, it was placed in a new 15 ml centrifuge tube and frozen at –80°C. After incubation for 15 minutes, IEF strips were placed in the single well of a 10 cm ReadyGel (BioRad) and sealed in place using liquid agarose solution. After polymerization, the gel was covered with a Tris SDS buffer and run in the second dimension at 100V for 1 hour and 30 minutes. No molecular weight standards were used. When the dye front had reached the bottom of the gel, current was removed and the gel removed from the apparatus. The gel was notched in the upper right corner and placed in modified Neuhoff staining solution overnight. After staining overnight, the gels were destained using several changes of 1% acetic acid solution with gentle agitation. After maximum visualization was produced, the gels were documented using the Nucleotech gel documentation system. (For COMPLETE protocol information, see the 23-page protocol composed by Jack Gallup, V PTH, spring 2000.)

No detectable differences were found between whole RBCs or RBC membranes incubated with STX as compared to those incubated with saline (control). These results were similar to those determined by one dimensional SDS-PAGE. These results are consistent with the mechanism of action of STX. Because RBCs lack cellular organelles necessary for cell intoxication (namely the golgi apparatus and the endoplasmic reticulum) they are likely protected from the direct effects of STX.

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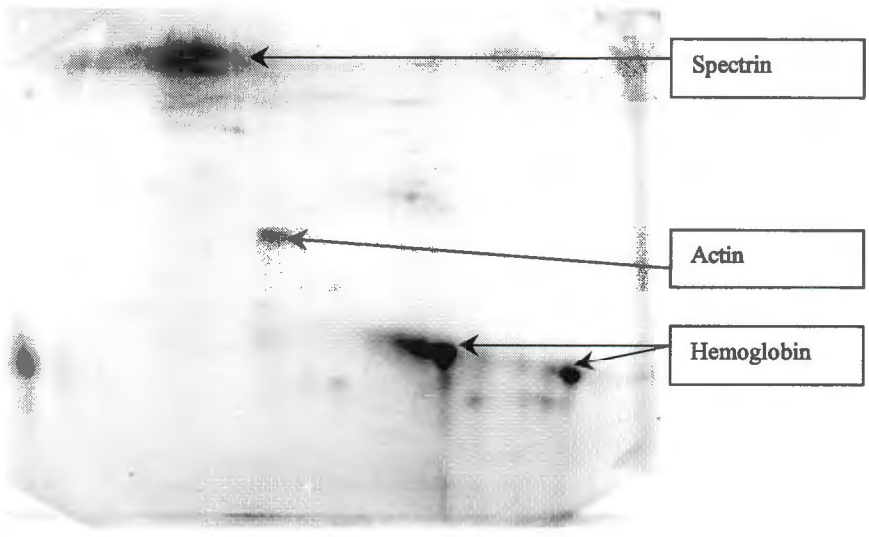

Figure 1: Two-dimensional electrophoresis (2D ELP) of control RBC membranes.

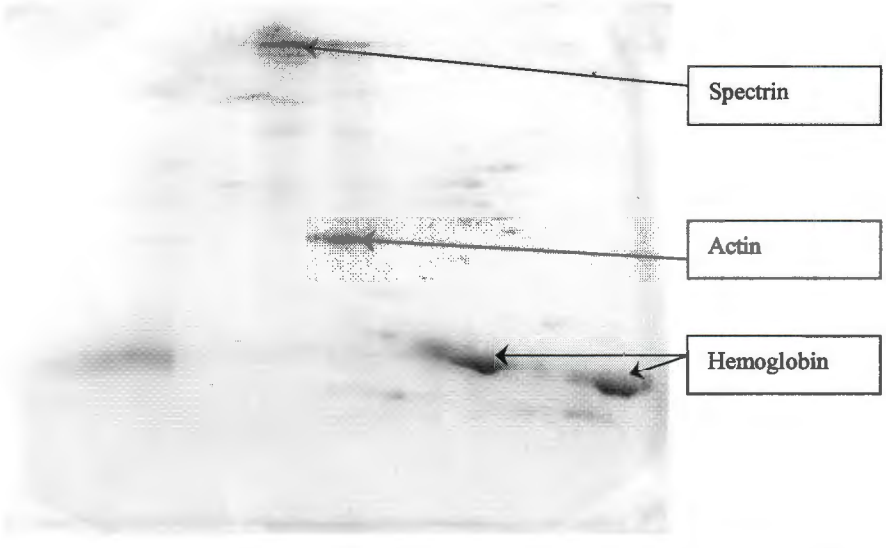

Figure 2: 2D ELP of RBC membranes after incubation with STX for 1 hour.

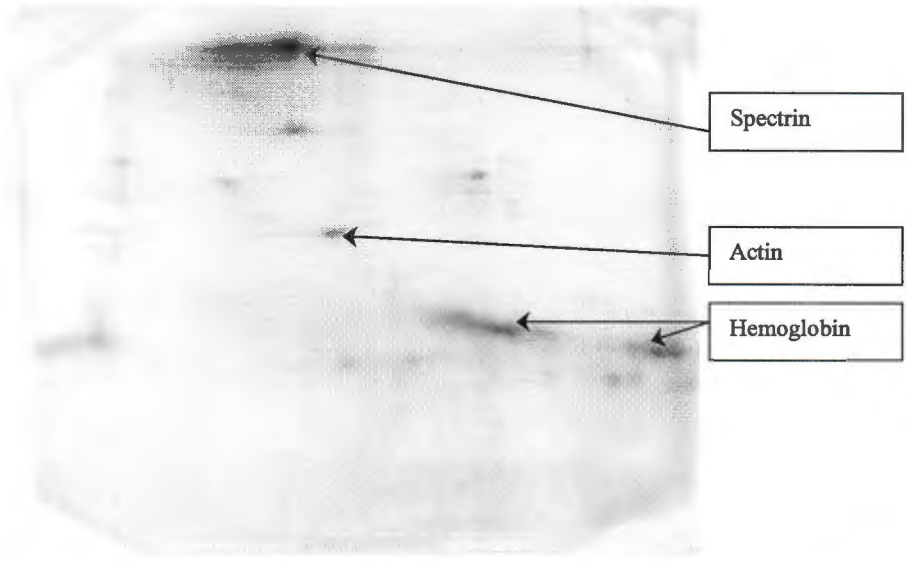

Figure 3: 2D ELP of RBC membranes after incubation with STX for 3 hours.

APPENDIX C:

PROTOCOLS

Erythrocyte Osmotic Fragility Protocol
(Reference: *Schalm's Veterinary Hematology*, 4th ed. (1986) pp 69-71)

Materials Needed:
5 ml pipetteman and 3 tips
20 µl pipetteman and 1 tip per assay
3 ml tubes (16 per assay)
NaCl stock solution
Ultrapure H_2O
50 ml centrifuge tubes (2)
Spectrophotometer cuvettes (17)

NaCl Stock Solution:
90 g NaCl
13.7 g Na_2HPO_4
1.9 g NaH_2PO_4
1 L Ultrapure H_2O
Adjust pH to 7.4
Solution is stable for months

1. Prepare 1% NaCl solution: Fill 50 ml centrifuge tube with 45 mls ultrapure H_2O. Using pipetteman, add 5 ml NaCl stock solution and mix. (50 mls 1% NaCl will be enough for two assays. Make fresh 1% solution if previous solution is older than two days.)

2. Have 50 mls of ultrapure H_2O available.

3. Using 5 ml pipetteman, prepare serial dilutions of saline in tubes labeled 1 – 16 using following chart (Use one tip for 1% NaCl and one for H_2O):

Tube #	**1% NaCl (mls)**	**H_2O (mls)**	**Tube #**
1	2.125	0.375	**1**
2	2.000	0.500	**2**
3	1.875	0.625	**3**
4	1.750	0.750	**4**
5	1.625	0.875	**5**
6	1.500	1.000	**6**
7	1.375	1.125	**7**
8	1.250	1.250	**8**
9	1.125	1.375	**9**
10	1.000	1.500	**10**
11	0.875	1.625	**11**
12	0.750	1.750	**12**
13	0.625	1.875	**13**
14	0.500	2.000	**14**
15	0.250	2.250	**15**
16	0.000	2.500	**16**

4. Turn on spectrophotometer and printer. Lamps must warm up for 30 mins.

5. Using 20 µl pipetteman, add 10 µl heparinized, well mixed whole blood to each tube. BE SURE TO WIPE THE TIP WITH A KIMWIPE BEFORE PLACING IN ITS SALINE TUBE!

6. Vortex each tube until cells are suspended and incubate at room temperature for 30 minutes. During incubation, locate and label 16 spectrophotometer cuvettes. Bring up "Absorbance/Transmittance" on spectrophotometer. Then load "osmotic fragility" program (Wavelength should be set at 540nm). On right side of screen, enter owner of calf, calf #, age, date, pre or post-innoculation. Fill extra cuvette with ultrapure H_2O for reference. Wait until lamps have been on for at least 30 mins before collecting reference.

7. Centrifuge all tubes at 2,000 rpm for 7 mins. While centrifuge is going, run reference by putting H_2O cuvette in position 1 and run "collect reference."

8. Without disturbing the cell button (if there is one), transfer supernatant from tube to its corresponding cuvette and place in cuvette holder in spectrophotometer (holds 8 samples at a time). Make sure that sample position is "1" then move down to auto run, enter 8 and press return. Spectrophotometer should automatically start reading each sample. After the first 8 samples have run, put in the next 8 samples, make sure that the sample position is "1" and then highlight "Auto Run."

   (Note: Supernatants in the later tubes should be red, while supernatants in early numbered tubes should be clear.)

9. After all 16 samples have been read, insert paper in the printer and highlight "print screen" and push return. Print 3 copies. (Try to keep the spectrophotometer readings as close to the top of the page as possible.)

10. Put all cuvetttes and tubes in Biohazard Waste. Turn off spectrophotometer, cover, and clean up.

Protocol For Human RBC Responses To *E. Coli* Toxins

RBC Preparation

1. Obtain human blood drawn by antecubital venipuncture into 3 ml EDTA and 3 ml lithium heparin tubes. Blood will be drawn by medical technologists in Clinical Pathology lab after signed, informed consent is obtained.

2. Tubes will be centrifuged at 3500 rpm for 5 mins and the plasma and buffy coats removed and disposed of.

3. RBCs will be washed three times in phosphate buffered saline. After washing, cells will be resuspended in PBS at a hematocrit of 25 – 30%.

4. 0.5 ml of cell suspension from both EDTA and heparin will be aliquoted into 4 12 X 75 mm tubes.

Toxin Preparation

1. Dilute 100 μgs SLT I with 100 μl PBS. Separate into 5 μl aliquots and freeze.

2. Dilute 1 μl of diluted toxin 1:4 with PBS to be used for 0.25 and 0.5 μgs assays.

RBC/Toxin Assay

1. Add 0.25, 0.5, and 1μgs of toxin to 0.5 ml of washed RBC suspension from both EDTA and heparinized samples. Vortex briefly to mix and incubate at 37°C for 30 mins.

2. For controls, PBS with no toxin will be incubated with washed RBC suspension (from EDTA and heparin) for 30 & 60 mins and at 24 hrs. at 37°C.

3. After incubation, a blood smear will be made from each tube and will be stained with Wright's stain and examined with a light microscope.

4. A wet mount of RBCs will also be made and examined with light microscopy.

5. Perform osmotic fragility after completion of incubation.

Protocol for RBC membrane protein preparation for SDS-PAGE
(Reference: Barker RN Electrophoretic analysis of erythrocyte membrane proteins and glycoproteins from different species. *Comp Hematol Int* 1:155-160, 1991.)

1. Samples were drawn into lithium heparin, sodium citrate has also been described as common anticoagulant

2. Whole blood was centrifuged for 10 mins at 3,500 rpm; the plasma and buffy coat removed (be aggressive in removing the buffy coat now, it will save time later)

3. Wash RBCs 6 times with PBS (pH 7.4), removing any residual buffy coat after each wash. Each wash consisted of adding 5 - 10 ml PBS to a tube containing ~2 ml RBCs, & mixing thoroughly. Centrifuge at 1,000 rpm for 1 – 2 mins (just enough to get a clear supernatant). Removed the supernatant and residual buffy coat and repeat.

4. RBCs lysed with 10 volumes of ice cold 20 mM Tris (pH 7.6) per one volume washed RBCs

5. Centrifuge at 30,000 rpm at 4°C for 30 mins.

6. The supernatant was removed and the membranes washed and centrifuged (as in step above) with the Tris solution until the supernatant was clear (usually about three times). Residual leukocytes and platelets appeared as white cell buttons and were removed with cotton-tipped applicators.

7. After preparation, RBC membranes were resuspended in the 20 mM Tris solution (just enough to get them all in suspension).

8. Preparations of RBC membranes were standardized by measuring absorbance at 540 nm and adding 20 mM Tris. RBC membrane solutions were aliquoted and frozen at –70°C until use.

Cell Culture Protocols

Media Preparation

Clonetics Endothelial Cell Growth Medium BulletKit (EGM)
Clonetics Microvascular Endothelial Cell Growth Medium BulletKit (EGM-MV)

500 ml Endothelial cell basal medium
10 μg/ml Human recombinant epidermal growth factor, (0.5 ml)
1.0 mg/ml Hydrocortisone, (0.5 ml)
50 mg/ml Gentamicin, 50 μg/ml Amphotericin-B, (0.5 ml)
3 mg/ml Bovine Brain Extract, (2 ml)
10 ml Fetal Bovine Serum (EGM) or 25 ml Fetal Bovine Serum (EGM-MV)
Prepare media in cell culture hood, aliquot into 100 ml sterile bottles. Store at 4°C.

Trypsin/Trypsin Inhibitor

Purchase 0.025% trypsin/0.01% EDTA and trypsin inhibitor pre-made. Thaw and aliquot into 5 mls volumes (use Sterile Corning 15 ml tubes). Perform this procedure in the cell culture hood using STERILE pipettes. Store at –70°C.

Phosphate Buffered Saline (pH 7.4)

16 g NaCl
0.4 g KCl
2.3 g Na_2HPO_4
0.4 g KH_2PO_4
1 L Ultrapure H_2O
Sterilize via 0.2 μm filtration into autoclaved bottles and store at 4°C (parafilm seal).

Receipt of Clonetics Proliferating Cells

1. Cells arrive in sealed flasks, filled completely with media. Check condition of cells.

2. After arrival, wipe exterior with alcohol and place in 37°C incubator, with room air supplemented with 5% CO_2. Incubate for 2 – 3 hours to equilibrate temperature.

3. Warm fresh media to 37°C (5 ml for T-25 flask, 10 ml for T-75 flask). Remove excess media from flask and discard. Aspirate excess media from interior of cap, dry cap with 70% alcohol (to prevent microbial contamination). Add fresh media.

4. Loosen the cap, return to incubator. Check cells and add fresh media in 24 hrs.

Setup for Cryopreserved Cell Culture

1. Plate microvascular and renal endothelial cells at a rate of 4 T-25 flasks per one cryovial. Plate other endothelial cells at a rate of 8 T-25 flasks per one cryovial.

2. Prepare sterile field by cleaning hood with 70% ethanol. Be sure that the hood fan has been operating for 30 minutes. Locate sterile, individually wrapped serologic pipettes and pipette-aid. ALL items placed in sterile field must be wiped with 70% ethanol.

3. In sterile field, transfer 5 mls warmed supplemented growth medium to each T-25 flask. Label with cell type, date, etc. Loosely place non-vented caps on flasks and allow to equilibrate at 37°C in incubator for 30 mins.

4. Locate micropipettor, STERILE tips and 37°C water bath.

Cryopreserved Cell Plating

Cryopreserved cells are very **fragile, handle as little as possible!!**

1. Remove cryovial from liquid nitrogen, wipe exterior with 70% ethanol. IN STERILE FIELD twist open cap of vial one-quarter turn to relive internal pressure and re-tighten.

2. Dip the bottom ¾ of the cryovial in 37°C water bath, and swirl gently for 1 to 2 minutes until contents are thawed. WATCH CLOSELY! Do not thaw cells for longer than 3 minutes.

3. Remove the cryovial immediately, wipe it dry, then with 70% ethanol and transfer to sterile field with warmed flasks waiting.

4. Note the color of the fluid in the thawed vial, cells from Clonetics should have pink cryo solution.

5. Remove the cap, DO NOT touch the interior threads!

6. Using a 1000 µl pipetter set to 800 µl, put the tip into the solution and slowly pipette up and down to resuspend the cells (no more than 5 times). Avoid making air bubbles by keeping the tip near the bottom.

7. Dispense equal volumes of cells into flasks. If plating four flasks, set pipettor to 250 µl, if plating eight flasks, set pipetter to 125 µl and dispense.

8. Replace cap (loosely) and rock flask to distribute cells without getting cap wet. Return flasks to incubator.

9. Check cells next morning, add fresh media. Most cells should be adherent.

Cell Maintenance

1. Examine cells DAILY! Wipe flasks with 70% alcohol before returning to incubator. If cells need new media, warm (37°C) only the amount needed prior to use. T-25 flasks usually use 5 – 7 mls, T-75 flasks usually use about 10 – 15 mls. More confluent flasks need larger amounts of media.

2. Change the growth media every other day, if cells are doing well. If cells are not doing well, change daily. Change the media by aspirating the old media with a STERILE Pasteur pipette attached to the vacuum line. Place the pipette in the corner AWAY from the adherent cells. Using a STERILE serologic pipette, aseptically transfer fresh media down the corner of the flask away from proliferating cells.

3. If desired, rinse cells with sterile, warmed PBS after removing old media and prior to adding fresh media. If there is evidence of bacterial contamination, rinse cells with PBS supplemented with antibiotics.

4. Healthy cells should have clear, non-granular cytoplasm, many mitotic figures and should have a rapid growth rate.

5. Cells should reach confluence in 5 – 7 days. Do not let cells get over confluent or they may undergo permanent contact inhibition.

Preparation for Subculture

1. Ensure that cell culture hood fan is running for 30 mins prior to use. Clean hood with 70% ethanol.

2. Thaw trypsin/trypsin inhibitor and warm to 37°C. Warm PBS to 37°C in water bath.

3. Mark new flasks with name, date and passage number and warm in 37°C incubator. Generally, one T-25 confluent flask should be split into 3 T-25 flasks or 1 or 2 T-75 flasks. One T-75 flask should be split into 3 T-75 flasks.

4. Warm fresh media in water bath. When warm, move to STERILE field and add 5 ml new media to T-25 flask or 10 ml to T-75 flask. Loosen caps and return flasks to incubator until use.

Subculture of Cells

**NOTE: Subculture of cells is a process that could result in death of cells if improperly done. Timing is CRITICAL!! Do NOT allow others to interrupt this process or dead cells may result.

1. Wipe flask of confluent cells with 70% ethanol and place in STERILE field. Place the appropriate number of warmed, equilibrated flasks in the hood after wiping with alcohol.

2. Aspirate old media using STERILE Pasteur pipette and vacuum bottle, keeping tip away from cells.

3. Rinse cells with warmed, sterile PBS, use 5 mls for T-25 flask and 10 mls for T-75 flask. Remove PBS with Pasteur pipette.

4. Use sterile serologic pipette, add 5 ml trypsin to T-25 flask, use 10 to 15 ml for T-75 flask. Note the time. Swirl to distribute solution.

5. Move the flask to the inverted microscope and observe. Cells should become loose and detach readily. Swirl contents occasionally to detach loose cells. Note the time, trypsinize cells ONLY for 3 to 5 minutes or as long as necessary to detach 95% of the cells. When most cells are loose, rap the flask on the palm of the hand to detach remaining cells.

6. IMMEDIATELY move to the cell culture hood and aseptically pipette trypsin inhibitor into the flask (use an equal volume to the amount of trypsin added). Swirl the flask.

7. Using a sterile serologic pipette, remove the detached cells in trypsin/trypsin inhibitor solution to a sterile centrifuge tube.

8. Aseptically add 5 to 10 mls of warmed, sterile PBS to the original flask and swirl to rinse any remaining cells into solution. Remove the PBS from the flask using a serologic pipette and place in the centrifuge tube with trypsin/trypsin inhibitor.

9. Centrifuge for 5 to 6 minutes at 2500 rpm. Remove promptly.

10. CAREFULLY aspirate the trypsin/trypsin inhibitor using a sterile Pasteur pipette attached to a vacuum bottle. DO NOT disturb the cell button!!!

11. Resuspend the cells in warmed media, use at least 1 ml for each flask to be seeded.

12. Using a sterile serologic pipette, aspirate the solution from the tube and divide the amount into the warmed, equilibrated flasks. Usually place 1 ml of cell suspension in each new flask (that already contains warmed, equilibrated media).

13. GENTLY swirl the flasks to distribute the cells and media WITHOUT getting solution into the cap. Loosen the cap and place in 37°C, 5% CO_2 incubator.

Because of the time sensitive nature of the subculture process, it is usually recommended that only one flask be subcultured at a time. However, with practice (and track shoes), a new flask of cells can be trypsinized while the previous flask is in the centrifuge.

Freezing of Cells

1. Perform trypsinization procedure as in the subculture process. Thaw sterile DMSO and have ready to use.

2. Instead of resuspending cells in media, resuspend the cells in a solution of equal parts of DMSO and growth media. DO NOT put one flask into one cryovial! Split the flask into at least 3 sterile cryovials of 1 ml each.

3. After the cells are in the cryovial, place the vial at –20°C until solid or overnight. Then move the vials to –70°C until the next day, then into liquid N_2.

4. If freezing part of the flask is desired along with subculture of part of the flask, be sure to split the trypsin/trypsin inhibitor solution into two centrifuge tubes PRIOR to centrifugation. Then resuspend cells in appropriate solution.

Protocol for EC/RBC Adhesion Experiments

1. Culture Human Umbilical Vein EC (HUVEC) on chamber slides until confluent. Add 1 μg STX-1; incubate at 37 for 1 hr. Can also use Human Renal Artery EC or Microvascular EC.

2. Collect heparinized whole blood and remove plasma, platelets and buffy coat. Wash cells 3 times in Hank's Balanced Salt Solution (HBSS) and resuspend to 3% PCV.

3. Remove media and toxin and wash HUVEC 3X with PBS.

4. Fill chamber with RBC suspension and incubate for 30 mins at 37.

5. Seal with tape, invert for 30 mins and remove gasket and chamber.

6. Stain, count numbers of RBCs attached to HUVEC

Protocol For STX Staining Of Endothelial Cells

1. Culture endothelial cells at 37°C with humidified air supplemented with 5% CO_2. Grow cells 1 well chamber slide covered with endothelial growth medium (with antibiotics) until confluent.

2. Incubate cells with 10 µl/well purified STX 1 for 24 hrs at culture conditions.

3. After incubation, wash cells with warmed medium 3 times.

4. Fix cells in cold (4°C) methanol for 5 minutes.

5. Air dry. Wash slides with PBS supplemented with 3% Bovine Serum Albumin (BSA). Do not dry.

6. Incubate slides with mouse 1:250 dilution (Prepare dilution in PBS-3%BSA) of anti-STX 1 IgG for 30 mins at 37°C.

7. Wash cells with PBS 3% BSA 3 times.

8. Cover slides with secondary antibody: mouse anti-IgG FITC. Dilute antibody 1:50 in PBS-3% BSA. Incubate in the dark for 2 hrs at room temp.

9. Wash with PBS-3% BSA 3 times for 10 mins each wash.

10. Cove slide with Vecta-Shield with propidium iodide (to stain the nuclei). Seal with Revlon nail polish. Observe with fluorescent microscope.

Two-Dimensional Electrophoresis Protocol

First Day
Sample Preparation
28 µl RBC membrane pellet (see previous)
413 µl Rehydration Buffer
49 µl DTE stock solution, add right before use
(0.08 g DTE in 2.5 ml rehydration buffer)

Rehydration Buffer
8M urea
2% CHAPS
1% IPG buffer from Amersham Pharmacia
A few grains of Bromophenol Blue

1. Add DTE stock solution to samples.

2. Pipette 125 µl protein solution to clean 7 cm ceramic trays. Avoid air bubbles! Remove plastic facing from IPG strip (pH 3 – 10 Linear, Amersham Pharmacia) and place in tray, gel side down. Cover with dry strip cover fluid or mineral oil and tray lid.

3. Place trays in IGphor and program to receive 2 hours rehydration followed by focusing at 25,000 to 30,000 V*hr. Usually takes about 24 hours.

Second Day

SDS Equilibration Buffer
6M Urea
30% Glycerol
2% SDS
Few grains of Bromophenol Blue
To each 10 mls of this solution, add 100 mg DTE just prior to use

Modified Neuhoff Stain
1 g G-250 Coomassie Blue
36 ml 85% Phosphoric Acid
340 ml Methanol
170 g Ammonium Sulfate
H_2O, q.s. to 1L

Agarose Sealing Solution
0.5% Agarose
Few grains of Bromophenol Blue
100 ml SDS running Buffer

1. Remove strips from trays, strips to be run in the second dimension place immediately in centrifuge tube with 10 mls of SDS equilibration buffer. Equilibrate for 15 minutes. If unable to run second dimension that day, place strips in 15 ml centrifuge tube and freeze at –80°C.

2. Boil 0.5% agarose sealing solution (with trace bromophenol blue) to liquify. Set up second dimension apparatus using BioRad 10 cm ReadyGels (8 – 16% gradient, 2D well).

3. Insert equilibrated gel strips into well and seal with slightly cooled sealing solution. Allow to polymerize.

4. Run gel for 1.5 to 2 hours at 100V on ice.

5. After dye front reaches the end of the gel, dismantle the apparatus; nick the gels to label and transfer to stain boats.

6. Add Modified Neuhoff stain, cover with plastic wrap and place on rotator. Stain overnight

Third Day

1. Remove modified Neuhoff stain, transfer to clean boats and add 1% acetic acid. Change solution several times until maximum visualization is achieved (usually about 2 – 3 hours.

2. Record image of gel using Nucleotech or other recording device.

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